Resistance of Very Young Mice to Inhaled Allergen Sensitization Is Overcome by Coexposure to an Air-Pollutant Aerosol

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The role of air pollution in the initiation of asthma is controversial. We sought to model the potential effects of air pollution on immune responses to inhaled allergens in developing lungs by using very young mice. Neonatal mice were repeatedly exposed to aerosolized ovalbumin (OVA; 3% in phosphate-buffered saline for 10 min/d, from Days 5 to 15 of age). Some mice were also exposed to leachate of residual oil fly ash (ROFA-s), a surrogate for ambient air particles, for 30 min, on Days 6, 8, and 10 of age). Repeated exposure of very young mice to allergen alone (OVA) or pollutant alone (ROFA-s) had no effect on airway hyperresponsiveness (AHR, measured as enhanced pause (Penh) with noninvasive plethysmography at Day 16 of age), and did not cause inflammation or OVA-specific antibody production. Similar exposures of adult mice to either OVA alone or to OVA + ROFA-s did result in AHR, without evidence of enhancement by combined exposure. In contrast, very young mice exposed to both OVA and ROFA-s showed significantly increased AHR (e.g., Penh with 50 mg/ml methacholine for OVA + ROFA-s versus OVA alone = 2.6 ± 0.4 [mean ± SE], versus 1.2 ± 0.1; $p < 0.01$, $n \geq 15$), and produced OVA-specific IgE and IgG upon allergen challenge a week later. Immunostaining of airways taken from mice at Day 11 showed a marked increase in Ia^+ cells after OVA + ROFA-s exposure. We conclude that exposure to pollutant aerosols can disrupt normal resistance to sensitization to inhaled allergens, and can thereby promote development of airway hypersensitivity in this neonatal/juvenile mouse model.

Asthma often begins in early childhood. Neonatal subjects, however, have an immature immune system and are predisposed to immune tolerance after repeated exposure to allergen (1, 2). One interpretation of this observation is that developing individuals need exposure to both allergen and some adjuvant-like cofactor to develop allergen sensitization. Cofactors associated epidemiologically with increased risk of developing asthma upon airway exposure include injurious agents such as infectious viruses (3) or passively inhaled tobacco smoke (4).

Air pollution, including increased levels of respirable particles, has been linked epidemiologically to increased manifestation of signs and symptoms in individuals with established asthma (5, 6). Since high amounts of particulate air pollution can also cause pulmonary injury (7, 8), it is reasonable to consider whether air particles can promote the initiation of asthma. Some epidemiologic studies report an association of air pollution levels with an increased incidence of asthma, whereas many others do not (9, 10). Experimentally, such components of air pollution as diesel exhaust particles (11, 12) and NO_2 (13) can act as adjuvants for stimulation of production of immunoglobulins, including IgE. Direct experimental testing of air pollution as an adjuvant for inhaled allergens may improve understanding of its role in asthma promotion, and may identify factors that merit additional epidemiologic or biologic study.

We performed such direct experimental testing with a rodent model of asthma. In mice, systemic sensitization (e.g., intraperitoneal injection of allergen protein with adjuvants) followed by exposure to aerosolized allergen leads to airway inflammation and hyperreactivity (14, 15). Repetitive exposure to aerosolized allergen alone, without systemic administration of allergen in adjuvant solutions, is arguably more representative of actual human interaction with allergens. Such airway sensitization can cause development of asthmalike features in mice, as described by Renz and coworkers (16). The latter and other investigators have generally used adult mice. Since asthma begins and is most problematic in early life, we performed studies with very young mice and compared the results with those obtained with adult mice. We found that in contrast to the case with adult mice, airway sensitization alone does not render neonatal mice asthmatic. This suggests important developmental differences in immune responses.

We also investigated the possible effects of air pollution on immune events seen in the developing lungs of young mice and in their adult counterparts. In these experiments we used aerosol exposure to leachate from residual oil fly ash (ROFA-s), a surrogate for ambient air particle pollution. In adults, exposure to this air pollutant aerosol alone or in concert with exposure to ovalbumin (OVA) increased allergen-specific immunoglobulin production but did not alter airway hyperresponsiveness (AHR). In contrast, exposure of neonatal/juvenile mice to OVA + ROFA-s overcame their lack of response to inhaled allergen alone, and resulted in effective airway sensitization to allergen.

METHODS

Animals

Newborn BALB/c mice were obtained commercially from Harlan Sprague-Dawley (Indianapolis, IN) as litters at Day 3 of age with their respective mother mice, or by in-house breeding. Each mother and litter was housed separately, fed a commercial pelletted mouse feed, and given water *ad libitum*. The mice were housed in an animal facility that was maintained at 22 to 24° C, with a 12-h dark/light cycle. Eight-week-old female BALB/c mice were also purchased and housed in the same manner as the litters.

Study Protocol

Airway sensitization and challenge with allergen. Figure 1 summarizes the experimental protocols used in the study. Mice were exposed to a nebulized aerosol of 3% (wt/vol) OVA (Grade III; Sigma Chemical, St. Louis, MO) in phosphate-buffered saline (PBS) (pH 7.4) from 5 to 15 d after birth (11 consecutive days), for 10 min/d within individual compartments of a mouse "pie" chamber (Braintree Scientific, Braintree, MA). The aerosol was generated with a Pari IS2 nebulizer (Sun Medical Supply, Kansas City, KS) connected to an air compressor (PulmoAID; DeVilbiss, Somerset, PA) (17). Control mice were exposed to PBS alone.

Adult mice (8 wk old) were also exposed to OVA or PBS in the

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same manner as the neonatal/juvenile mice. After sensitization, physiologic testing (described in detail subsequently) was performed 24 h after the last OVA exposure (16 d after birth, or at approximately 10 wk of age for adult mice). In addition to the sequence just described, physiologic analysis was repeated on some groups of mice at 1 wk after the initial physiologic analysis following sensitization (Figure 1). In these experiments, all groups of mice were then exposed to a nebulized aerosol of 3% (wt/vol) OVA for three consecutive days (Days 24 to 26 after birth) for 10 min on each day. Analyses following allergen challenge included physiologic testing after 24 h and pathologic evaluation after killing at 48 h (Days 27 and 28, respectively) after the last OVA exposure.

Coexposure to aerosolized ROFA-s. A single large, batch of residual oil fly ash (ROFA) was obtained from the precipitator unit of a local power plant. The ROFA was suspended (100 mg/ml in PBS) and sonicated for 10 min. After being allowed to rest for 30 min at room temperature (RT), the ROFA suspension was incubated with rotation at 37° C for 4 h, and was then centrifuged at 3,700 rpm (3,000 × *g*) for 10 min. The supernatant (ROFA-s) was removed and used for aerosol exposure after 1:1 (vol/vol) dilution in PBS. The metal and elemental composition of ROFA-s was analyzed through inductively coupled plasma mass spectroscopy, using a sample prepared in a manner identical to that for the initial batch of ROFA-s except for the use of analytical grade H_2O as diluent. The ROFA-s was found to contain the following constituents, in order of concentration (in μg/ml): Ni, 341.2; V, 323.4; Zn, 232.3; Co, 18.3; Mn, 15.8; Ca, 8.4; Mo, 6.7; Sr, 6.1; Mg, 5.0, Sb, 0.9, Cd, 0.6; total mass = 0.958 mg/ml). No iron was detected in the supernatant produced from the ROFA sample. Groups of mice were treated with OVA or PBS delivered by the nebulizer system described earlier, and were exposed to aerosolized ROFA-s at 6, 8, and 10 d after birth for 30 min/d, just before their daily OVA/PBS exposure. The flow rate for the nebulizer was 4.6 L/min. On the basis of the total mass of ROFA-s in 15 ml of nebulized solution, and a delivery period 30 min, and assuming a total ventilation of 6 ml/min per 5 g young mouse (18) and a 5% deposition fraction for the aerosol, one can estimate a total lung exposure per aerosol exposure of 18 μg, and a total deposition of 0.9 μg of the metals and other elements present in ROFA-s. For adult mice, the corresponding estimates are 75 μg and 4 μg, respectively.

In adult mice, exposure to ROFA-s took place on Days 2, 4, and 6 of daily OVA/PBS exposure. In pilot studies, aerosol exposure of adult mice to this concentration of ROFA-s for a period of 30-min caused transient, mild pulmonary inflammation, manifested by neutrophils in bronchoalveolar lavage fluid (BALF) after exposure (% neutrophils: 1.39 ± 0.33%, 7.10 ± 1.22%, 1.97 ± 0.37%, and 1.48 ± 0.26% [mean ± SEM] at 6, 12, 24, and 48 h, respectively; n ≥ 5 mice in each group).

Airway sensitization and maturation. To investigate whether airway sensitization or allergen-specific immune tolerance occurs in response to repetitive exposure to aerosolized allergen, we left some neonatal/juvenile mice untreated for 2 wk after the initial airway sensitization (Days 5 to 15 after birth; Figure 1). We then exposed these animals once again to OVA/PBS on a daily basis for 11 consecutive days. After this second airway sensitization, these mice were analyzed according to the same protocol as that used with adult mice.

Physiologic Analysis

Airway responsiveness of mice to increasing concentrations of aerosolized methacholine (MCh) was measured through a whole-body plethysmography system (Buxco, Sharon, CT). Briefly, each mouse was placed in a chamber, and continuous measurement of the box pressure–time wave was made via a transducer connected to a computer data-acquisition system. The main indicator of airflow obstruction, enhanced pause (Penh), which shows strong correlation with airway resistance as measured according to standard evaluation methods, was calculated from the box pressure–time waveform (19). After measurement of baseline Penh, either aerosolized PBS or MCh in increasing concentrations (6, 12, 25, 50, and 100 mg/ml) was nebulized through an inlet of the chamber for 1 min, and measurements of Penh were made for 9 min after each dose. Penh values for the first 5 min after each nebulization were averaged and used to compare results across treatment groups and among individual mice.

Pathologic Analysis

After physiologic testing in airway-sensitized or allergen-challenged mice, the animals were euthanized with sodium pentobarbital (Veterinary Laboratories, Lenexa, KS). The chest wall was opened and the animals were exsanguinated by cardiac puncture. Serum was prepared and stored at −20° C. The trachea was cannulated after blood collection. Bronchoalveolar lavage (BAL) was performed five times with 0.3 ml (after sensitization) or with 0.4 to 0.6 ml (after allergen challenge) of sterile PBS instilled and harvested gently in each wash. Lavage fluid (recovery volume was about 90% of the instilled volume) was collected and centrifuged at 1,200 rpm (300 × *g*) for 10 min, and the cell pellet was resuspended in 0.5 ml PBS. Total cell yield was quantified with a hemocytometer. Differential counts of BAL cells made on cytocentrifuge slides prepared by centrifugation of samples at 800 rpm for 5 min (Cytospin 2; Shandon, Pittsburgh, PA). These slides were fixed in 95% ethanol and stained with Diff-Quik (American Scientific Products, San Diego, CA) (a modified Wright–Giemsa stain), and a total of 200 cells in each sample were counted by microscopy. Macrophages, lymphocytes, neutrophils, and eosinophils were enumerated. After lavage, the lungs were instilled with 10% buffered formalin, removed, and fixed in the same solution. After paraffin embedding, sections for microscopy were stained with hematoxylin and eosin.

Assay of OVA-Specific Immunoglobulin

Anti–OVA–specific IgE antibody was measured with an enzyme-linked immunosorbent assay (ELISA) (20, 21). Ninety-six–well microtiter plates (Nunc, Rosskilde, Denmark) were coated with 0.2 μg of monoclonal rat antimouse IgE (Pharmingen, San Diego, CA) diluted in 0.1 M carbonate buffer (pH 9.5). After overnight incubation at 4° C, plates were washed with PBS–Tween 0.05% and blocked with PBS–bovine serum albumin (5% [wt/vol], pH 7.4) for 1 h, after which

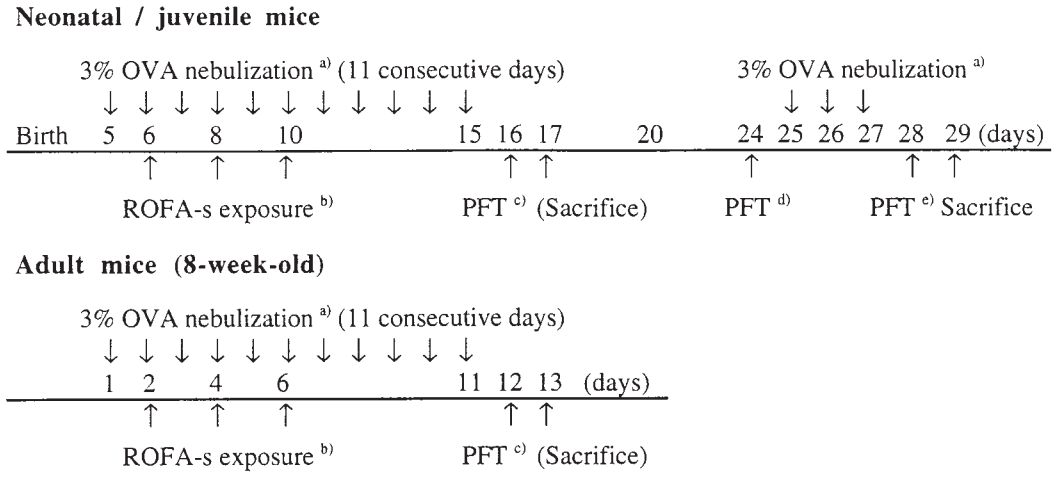

Figure 1. Schematic summary of experimental protocols used in study. (a) Exposed to 3% OVA (w/v) in PBS (pH 7.4) for 10 min/d. (b) Exposed to ROFA leachate (50 mg/ml) in PBS for 30 min/d. (c–e) Pulmonary function testing; (c) post sensitization, (d) pre- and (e) post-allergen challenge.

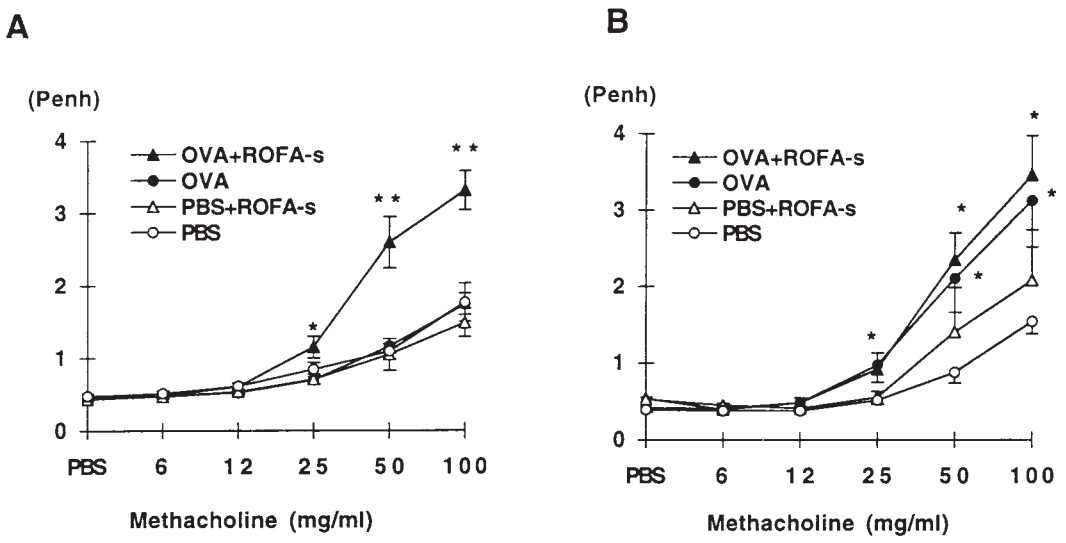

Figure 2. (*A*) Physiologic testing with noninvasive plethysmography (measured as Penh) in neonatal/juvenile mice after exposure to allergen (OVA), ROFA-s, or OVA + ROFA-s according to the protocol shown in Figure 1, *top* and detailed in text. AHR was seen only in mice exposed to both allergen and pollutant (OVA + ROFA-s). $^{**}p < 0.01$, $^{*}p < 0.05$ versus all other groups; $n \geq 16$ mice/group. (*B*) Protocol identical to that in (*A*), used with adult mice, results in AHR in mice exposed to allergen alone (OVA) or to allergen and pollutant (OVA + ROFA-s). $^{*}p < 0.05$ versus PBS or PBS + ROFA-s groups; $n \geq 6$ mice/group. Exposure to OVA and ROFA-s did not significantly enhance the response over that to OVA alone in adult mice.

serum samples were added. After overnight incubation at RT and washing, biotinylated OVA (1 μg/ml) was added to the plates. Plates were incubated for 1 h at RT and washed once again. After another 1-h incubation, with streptavidin–horseradish perioxidase (1:4,000; Zymed, San Francisco, CA), the reaction chromogen was generated with trimethylbenzidine substrate (TMB One-Step; Dako, Carpinteria, CA). Plates were read in a Softmax plate reader (Molecular Devices, Menlo Park, CA) at 450 nm. Serum pooled from OVA-sensitized and airway-challenged adult mice (in which AHR had already been confirmed) was used as a positive control. For each assay, values for serum samples were normalized relative to the absorbance of positive control serum, after subtraction from both the sample and control values for a blank solution containing buffer only. The results are expressed as indices (ratio of test serum to positive control serum).

As with the IgE antibody, anti-OVA–specific IgG antibody was also measured with ELISA. Briefly, plates were coated with OVA (10 μg/ml) by overnight incubation (4° C) and washed, and serum samples were incubated for 1 h at RT. After washing of samples with PBS–Tween 0.05%, peroxidase-conjugated goat antimouse IgG (1/2,000; Jackson Research, West Grove, PA) was added for 1 h. After a final wash, TMB was added as substrate. Quantitation of changes in absorbance, and data analysis, were performed as described earlier.

Immunohistochemical Study of Ia^+ Cells in the Trachea

Some mice were killed at 11 d after birth (the day immediately after the last coexposure to OVA/PBS and ROFA-s. The trachea was removed, covered with ornithyl carbamyltransferase embedding compound (Miles, Elkhart, IN), frozen promptly, and kept at −70° C. Immunohistochemical staining of cryostat sections (8 μm) with rat monoclonal antimouse Ia antibody (Pharmingen) was done with the peroxidase–antiperoxidase (PAP) method. Briefly, slides were air-dried, fixed in both 2% paraformaldehyde (10 min) and 100% methanol (10 min), and incubated with 5% goat serum (10 min) in PBS. Each of these steps was followed by washing in PBS–Tween 0.05%. After overnight incubation of the slides with monoclonal anti-Ia antibody (1 μg/ml) at 4° C, the second antibody (goat antirat IgG), and rat PAP (both from Sternberger Monoclonals, Lutherville, MD), were applied sequentially, followed by 1-h incubations at RT. Localization of Ia was revealed with diaminobenzidine as substrate and hematoxylin as a nuclear counterstain. Slides were evaluated light microscopically and Ia^+ cells in the tracheal mucosa (including epithelia and fibrocartilage layers) were counted. The cell counts were adjusted according to the number of cartilage rings present in the counting area, in order to normalize for tracheal sample length. Tracheal tissues of adult mice after 5 d exposure to OVA/PBS, and of naïve 8-wk–old mice housed under specific-pathogen free conditions, were used as controls.

Statistical Analysis

Data are expressed as mean ± SEM. Data were elevated through analysis of variance. A value of $p < 0.05$ was considered significant. Data analyses were done with Statview version 4.5 statistical software (Abacus Concepts, Berkeley, CA).

TABLE 1

BRONCHOALVEOLAR LAVAGE CELL FINDINGS AFTER AIRWAY SENSITIZATION IN NEONATAL MICE

	OVA + ROFA-s	OVA	PBS + ROFA-s	PBS
Total cells, $\times 10^5$/ml	1.19 ± 0.12	1.44 ± 0.30†	1.27 ± 0.99	1.04 ± 0.96
Macrophages, %	91.91 ± 0.12*	96.60 ± 0.43†	98.40 ± 0.10	98.78± 0.16
Lymphocytes, %	0.67 ± 0.68	0.70 ± 0.17	0.50 ± 0.16	0.67 ± 0.14
Neutrophils, %	6.58 ± 0.31*	1.55 ± 0.51‡	0.50 ± 0.16	0.22 ± 0.09
Eosinophils, %	0.83 ± 0.28	1.15 ± 0.39‡	0.60 ± 0.29	0.33 ± 0.12
Body weight, g	5.74 ± 0.12	5.35 ± 0.30	5.52 ± 0.20	5.51 ± 0.28
n	6	10	5	9

Definition of abbreviations: OVA = ovalbumin; PBS = phosphate-buffered saline; ROFA = residual oil fly ash.

* $p < 0.01$ versus all other groups.

† $p < 0.01$ versus PBS group.

‡ $p < 0.05$ versus PBS group.

TABLE 2

BRONCHOALVEOLAR LAVAGE CELL FINDINGS AFTER AIRWAY SENSITIZATION IN ADULT MICE

	OVA + ROFA-s	OVA	PBS + ROFA-s	PBS
Total cells, $\times 10^5$/ml	1.00 ± 0.04*	0.67 ±0.06	1.00 ± 0.06*	0.50 ±0.04
Macrophages, %	97.75 ± 0.36	97.25 ± 0.74†	98.08 ± 0.33	98.79 ± 0.13
Lymphocytes, %	1.33 ± 0.21	2.00 ± 0.45†	1.67 ± 0.33	0.96 ± 0.16
Neutrophils, %	0.33 ± 0.17	0.79 ± 0.41	0.25 ± 0.11	0.25 ± 0.10
Eosinophils, %	0.58 ± 0.20‡	0.08 ± 0.06	0.00 ± 0.00	0.00 ± 0.00
Body weight, g	17.93 ± 0.31	17.67 ± 0.46	17.84 ± 0.42	18.21 ± 0.46
n	6	12	6	12

Definition of abbreviations: OVA = ovalbumin; PBS = phosphate-buffered saline; ROFA = residual oil fly ash.
* $p < 0.01$ versus OVA and PBS groups.
† $p < 0.05$ versus PBS group.
‡ $p < 0.01$ versus all other groups.

RESULTS

Airway Sensitization in Neonatal/Juvenile versus Adult Mice

After exposure to aerosolized OVA alone for 11 consecutive days, neonatal/juvenile mice (16 d old when tested) did not manifest AHR upon MCh challenge (Figure 2A). However, mice exposed to OVA and ROFA-s (OVA + ROFA-s group) showed in a significant increase in AHR to MCh at concentrations over 25 mg/ml. This AHR was not seen in control groups exposed to PBS or ROFA-s alone. The solution of ROFA-s in PBS used in the study was acidic (pH 5.5); however, pH-adjusted ROFA-s (pH = 7.2 to 7.4, n = 10) produced the same results, whereas aerosols of acidic (pH 5.5) vehicle (PBS) had no effect (data not shown; n = 5). Analysis of BALF showed a small increase in neutrophils in the lungs of the OVA + ROFA-s group as compared with other groups of mice (Table 1; $p < 0.01$). No increase in total cell number or eosinophils was observed in the OVA + ROFA-s group. The growth rate among the four treatment groups, as reflected by body weight, did not differ (Table 1).

In contrast to neonatal/juvenile mice, adult mice, after identical exposure to aerosolized OVA for 11 consecutive days, did show AHR to MCh (Figure 2B). Further exposure, to ROFA-s, did not affect these animals' airway responsiveness: Penh data for OVA + ROFA-s mice were superimposable on those of the OVA-exposed mice. As in the findings by Renz and coworkers (16), there was no apparent inflammatory cell recruitment within airway tissues on histopathologic examinations (not shown), nor were significant increases in eosinophils detected in BALF from OVA-exposed adult mice (Table 2). Adult mice exposed to both OVA and ROFA-s showed a statistically significant but quite small increase in BAL eosinophils (< 1%; *see* Table 2).

Analyses before and after Allergen Challenge

One week after the initial testing, retesting of airway responsiveness was performed. The AHR that had been seen in the OVA + ROFA-s group after airway sensitization was no longer detected, and there was no physiologic difference among the groups (prechallenge; Figure 3A). After three consecutive days of exposure to allergen (OVA), only the group initially exposed to OVA + ROFA-s again exhibited AHR. In contrast, there was no change in the minimal airway responsiveness of the other three groups (Figure 3B). BALF analysis showed a small increase in total cell number in the OVA + ROFA-s group ($p < 0.05$ versus other groups), without significant changes in cell differential numbers, including those of eosinophils (Table 3). Histologically, there was no apparent inflammatory response in the airways or lungs of the groups exposed to PBS, PBS + ROFA-s, or OVA alone. Some mice (five of 29 examined) in the OVA + ROFA-s group showed slight pulmonary tissue abnormalities, consisting mainly of perivascular accumulations of mononuclear cells (Figure 4).

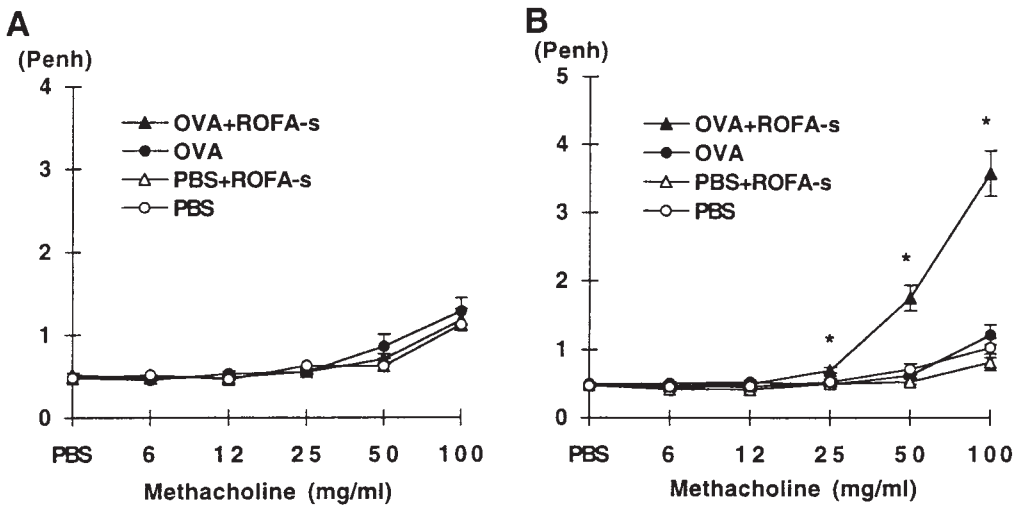

Figure 3. Effect of airway sensitization on subsequent response to allergen challenge. As indicated in Figure 1, a cohort of neonatal/juvenile mice subjected to 11 consecutive days of aerosol exposure to allergen with or without ROFA-s were rested for 1 wk before challenge with 3 d exposure to OVA. Physiologic testing on the day before the OVA challenge (*A*) showed minimal AHR in all four groups. Repeat testing after allergen challenge (*B*) caused AHR only in mice exposed to both allergen and pollutant (OVA + ROFA-s) during the initial airway sensitization period. *$p < 0.05$ versus all other groups; $n \geq 11$ mice/group.

TABLE 3

BRONCHOALVEOLAR LAVAGE CELL FINDINGS AFTER OVALBUMIN CHALLENGE IN NEONATAL/JUVENILE MICE SUBJECTED TO AIRWAY SENSITIZATION PROTOCOL

	OVA + ROFA-s	OVA	PBS + ROFA-s	PBS
Total cells, $\times 10^5$/ml	0.92 ± 0.05*†	0.74 ± 0.39	0.71 ± 0.08	0.59 ± 0.04
Macrophages, %	96.94 ± 0.48	96.22 ± 1.31	99.18 ± 0.21	97.32 ± 0.50
Lymphocytes, %	0.78 ± 0.10	1.17 ± 0.25	0.59 ± 0.11	1.00 ± 0.22
Neutrophils, %	1.58 ± 0.40	0.76 ± 0.22	0.18 ± 0.16	1.59 ± 0.49
Eosinophils, %	0.71 ± 0.14	1.65 ± 1.00	0.05 ± 0.05	0.18 ± 0.08
Body weight, g	9.38 ± 0.39	10.13 ± 0.25	9.02 ± 0.46	9.79 ± 0.27
n	45	27	11	11

Definition of abbreviations: OVA = ovalbumin; PBS = phosphate-buffered saline; ROFA = residual oil fly ash.
* $p < 0.05$ versus PBS 1 ROFA-s group.
† $p < 0.01$ versus OVA and PBS groups.

Analyses of OVA-Specific Immunoglobulin

After the initial airway sensitization period in juvenile mice, no OVA-specific IgE was detected in serum from animals killed at this time point (16-d–old mice). However, OVA-specific IgG production was detected, but only in OVA + ROFA-s–exposed mice (Figures 5A and 5B). In mice (re-)challenged with allergen after a 1 wk rest interval (29-d–old mice), OVA-specific IgE was detectable and was significantly increased in the OVA + ROFA-s group (Figures 5C and 5D). Although OVA-specific IgG was detected in both the OVA and OVA + ROFA-s groups, it was present in significantly higher concentration in the latter group. In contrast, adult mice had detectable OVA-specific IgG after initial airway sensitization (repetitive exposure for 11 d). In addition, adult mice exposed to OVA + ROFA-s had significantly higher levels of OVA-specific IgG than did juvenile mice (Figure 6B).

Immunohistochemistry of Tracheal Mucosa

We sought to determine whether coexposure to OVA and ROFA-s results in increased numbers of antigen-presenting cells in the airways. We enumerated Ia^+ cells in the airways of 11-d–old mice treated according to the protocol shown in Figure 1. Some Ia^+ cells, especially those within the tracheal epithelia, showed the characteristic appearance of dendritic cells (Figure 7A). In the OVA + ROFA-s group, a significantly higher number of Ia^+ cells was seen in the tracheal mucosa (Figure 7B), indicating enhanced recruitment of antigen-presenting cells (including dendritic cells) to the airways.

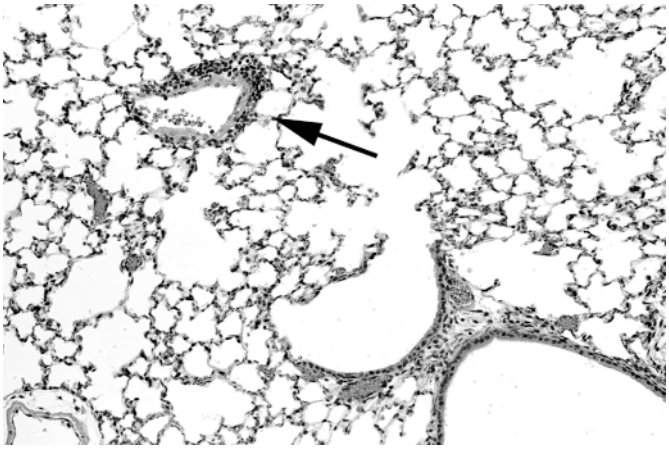

Figure 4. Histopathologic analysis of lung tissue from neonatal/juvenile mice after exposure to both allergen and ROFA-s aerosols showed occasional perivascular mononuclear infiltrates (*arrow*) and minimal airway inflammation (original magnification: ×200).

Analysis of a Second Airway Sensitization Trial after Maturation

In contrast to results with adult mice, the initial trial of airway sensitization to OVA did not succeed in neonatal/juvenile mice. To determine whether these mice had developed tolerance to OVA, we reexposed a cohort to OVA after a 2-wk rest interval. After the second trial of 11 d of aerosol exposure to allergen or control PBS, starting at 30 to 32 d of age, the OVA-exposed group (OVA/OVA group) showed AHR (Figure 8A) and increased numbers of eosinophils in their BALF (Table 4). After this second airway sensitization, OVA-specific IgE was not detected (Figure 8B). OVA-specific IgG was detected in mice exposed twice to OVA, and to a lesser degree in mice exposed initially to OVA and then to PBS in the second trial (Figure 8C). These results indicate that the resistance to airway sensitization observed in very young mice did not stem from permanent tolerance to the OVA used as allergen.

DISCUSSION

There is abundant epidemiologic evidence that air pollution causes exacerbation of existing asthma (5, 9, 22). In contrast, whether particles or other components of air pollution can promote initiation of asthma is controversial (9, 10). The major findings of the present study were that: (*1*) very young mice, in contrast to adult mice, are resistant to allergic sensitization by repeated exposure to aerosolized allergen; and (*2*) exposure of very young mice to both an aerosolized pollutant and to an allergen overcomes this normal resistance and results in effective airway sensitization.

Our results show that airway sensitization alone was not effective for producing allergy in neonatal mice, but that dual exposure, to allergen and ROFA-s, resulted in airway allergy. Although mice exposed to both OVA and ROFA-s (OVA + ROFA-s group) showed no apparent eosinophil recruitment to the airways or lungs, they did manifest AHR upon MCh challenge, and low levels of allergen-specific IgG were detected in their sera. AHR was diminished after a 1 wk rest interval. Notably, when this rest period was followed by 3 d of allergen challenge, only the mice exposed to both OVA and ROFA-s (OVA + ROFA-s group) again showed AHR. This result demonstrated that these mice (OVA + ROFA-s group) had developed immunologic memory for the allergen to which they had been exposed. Moreover, this (re-)challenged group of mice showed both allergen-specific IgE and IgG, confirming that initial exposure caused immune sensitization to allergen. We interpret these data as showing that ROFA-s acted as

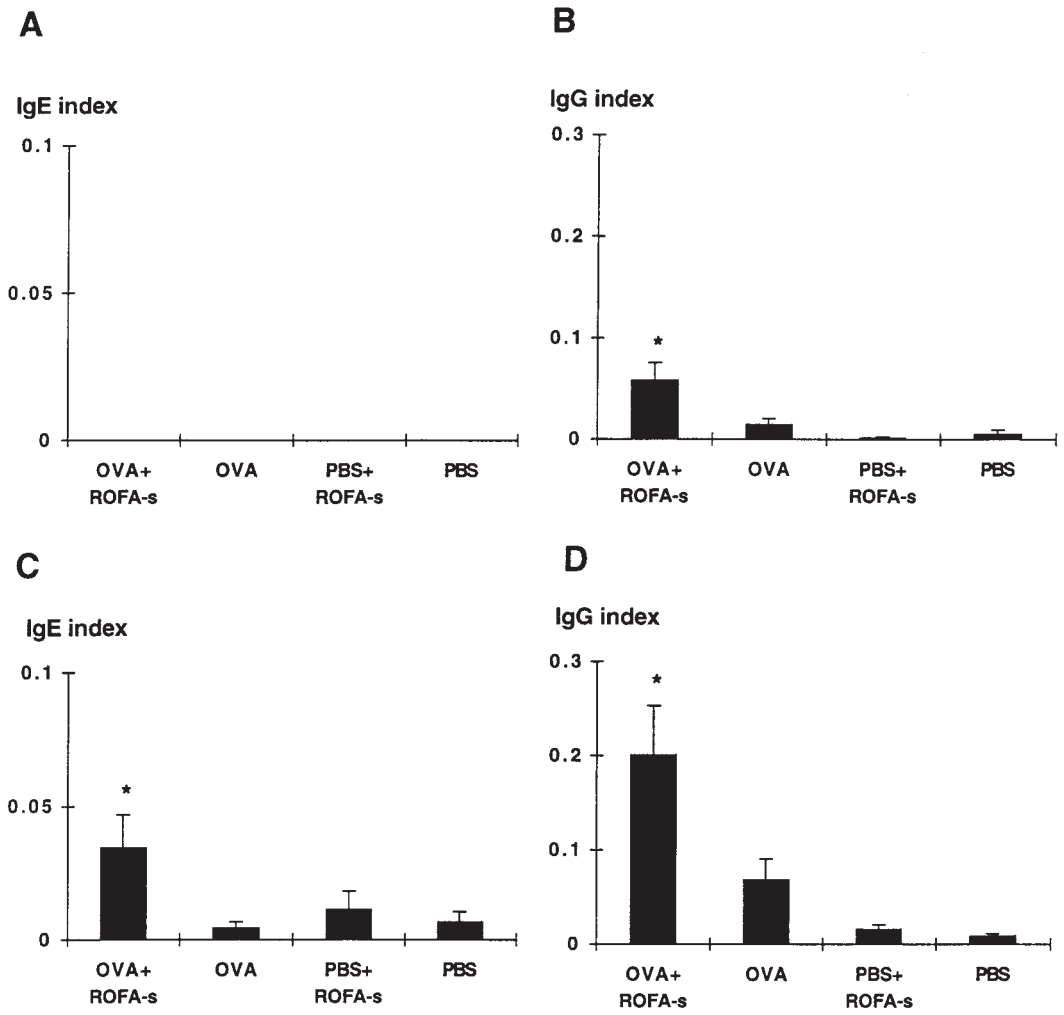

Figure 5. Serologic analysis of allergen-specific immunoglobulins in juvenile mice. After the initial airway sensitization period, all groups were negative for OVA-specific IgE (*A*), whereas OVA-specific IgG was significantly increased in the (OVA + ROFA-s–exposed group (*B*). *$p < 0.05$ versus all other groups; $n \geq 5$ mice/group. In a cohort of mice rested for 1 wk and then challenged with OVA (3 d of aerosol exposure), both OVA-specific IgE (*C*) and OVA-specific IgG (*D*) were significantly increased in the (OVA + ROFA-s–exposed) group. *$p < 0.05$ versus all other groups, $n \geq 6$ mice/group.

an adjuvant and promoted airway sensitization in mice also exposed to OVA.

The basis for the lack of airway sensitization in neonatal/juvenile as compared with adult mice is unknown. Generally, the immune response in the perinatal and neonatal periods is not the same as in mature individuals (23, 24). It is also known that repetitive exposure to inhaled allergen early in life can cause immune tolerance (2). We sought to specifically investigate whether the absence of sensitization in the very young mice that we exposed to daily allergen aerosols could be explained by induction of tolerance. The ability of a second exposure to aerosolized allergen to cause allergic airway inflammation, AHR, and increased allergen-specific IgG argues against this possibility.

Another possible mechanism for the diminished or absent

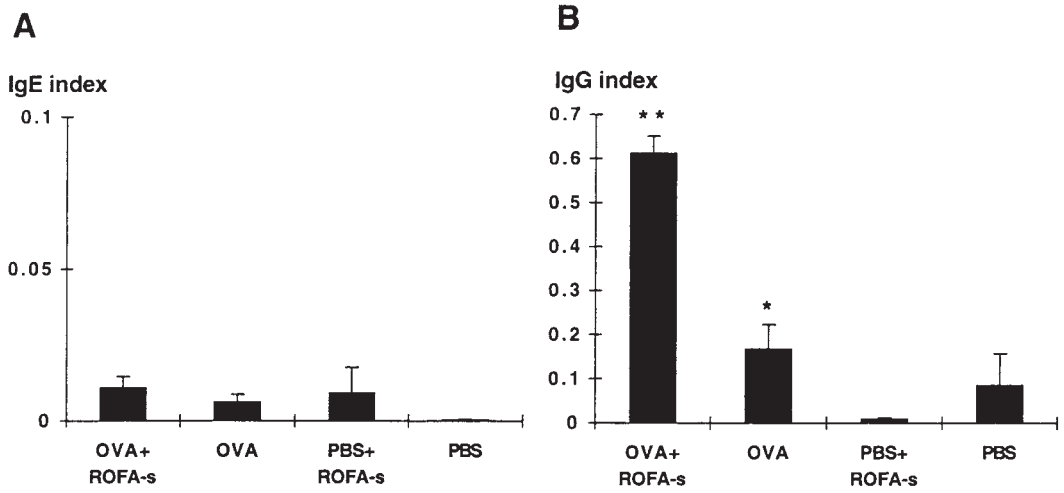

Figure 6. Serologic analysis of allergen-specific immunoglobulins in adult mice. After the initial airway sensitization period, no significant increase in OVA-specific IgE (*A*) was seen in any group as compared with the PBS control group ($p \geq 0.05$ versus all other groups), whereas OVA-specific IgG (B) was significantly increased in the OVA + ROFA-s–exposed and allergen (OVA)-only groups. **$p < 0.05$ versus all other groups, $n = 6$ mice/group; *$p < 0.05$ versus both control groups (PBS alone and PBS + ROFA-s); $n = 6$ mice/group.

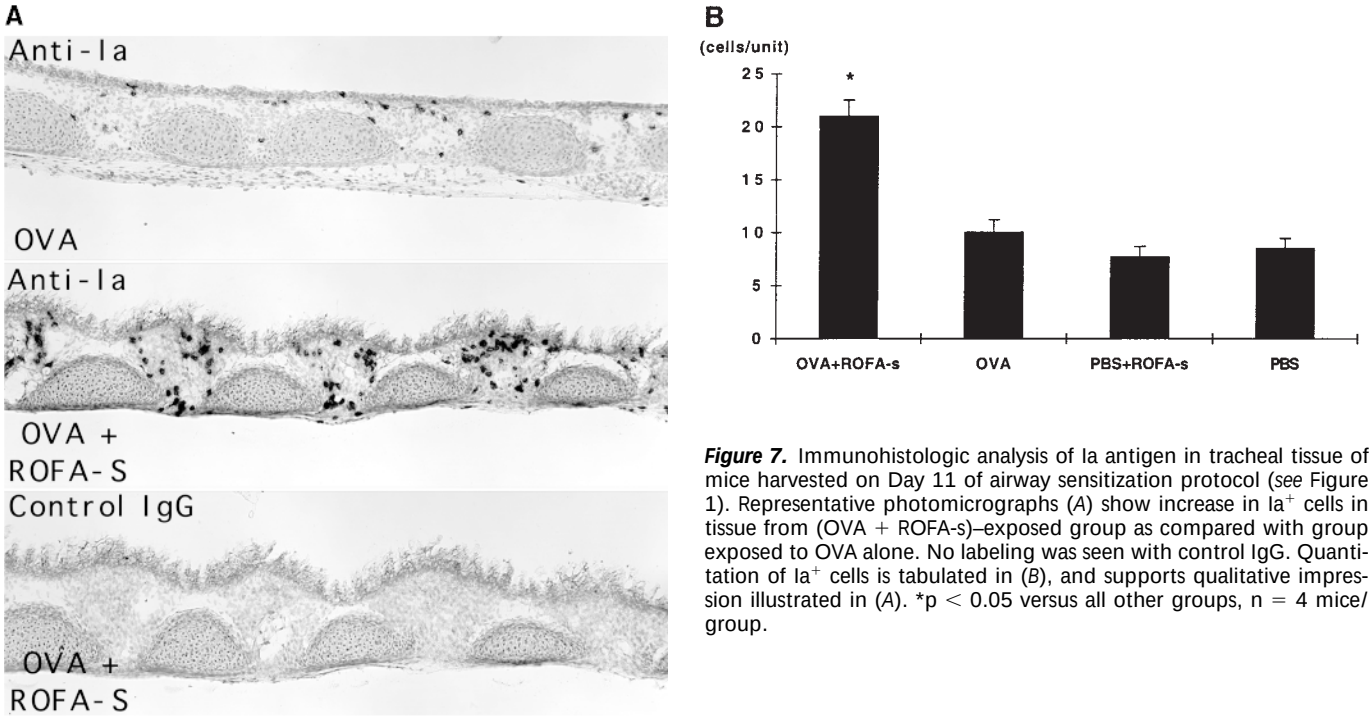

Figure 7. Immunohistologic analysis of Ia antigen in tracheal tissue of mice harvested on Day 11 of airway sensitization protocol (*see* Figure 1). Representative photomicrographs (*A*) show increase in Ia^+ cells in tissue from (OVA + ROFA-s)–exposed group as compared with group exposed to OVA alone. No labeling was seen with control IgG. Quantitation of Ia^+ cells is tabulated in (*B*), and supports qualitative impression illustrated in (*A*). *$p < 0.05$ versus all other groups, $n = 4$ mice/group.

response to airway sensitization in very young mice may involve recruitment or function of antigen-presenting cells. Dendritic cells are crucial for development of allergic sensitization (25), and are recruited to the airway tissues promptly after injury caused by radiation or infection in adults (26). Relatively few dendritic cells are observed in the airways very early in life; the numbers of these cells increase during development (27). ROFA can cause airway epithelial injury and lung inflammation via metal-dependent oxidant injury, induction of cytokine expression, and stimulation of prostaglandin production *in vitro* and *in vivo* (28–30). We tested the effect of exposure to ROFA-s on dendritic cell number in the airways of mice in our protocol. Because no uniquely dendritic cell-specific antibody is available, dendritic cells are recognized by a combination of morphologic and immunophenotypic (e.g., expression of Ia antigen) criteria. The number of Ia^+ cells was increased in the airways of juvenile mice (11 d old) exposed to OVA + ROFA-s and examined 1 d after the third exposure to aerosolized ROFA-s (Figure 7). Few Ia^+ cells were seen in the tracheal tissues of 11-d–old mice exposed to PBS or PBS + ROFA-s aerosols. This indicates that airway injury by ROFA-s alone did not cause recruitment of Ia^+ cells to the

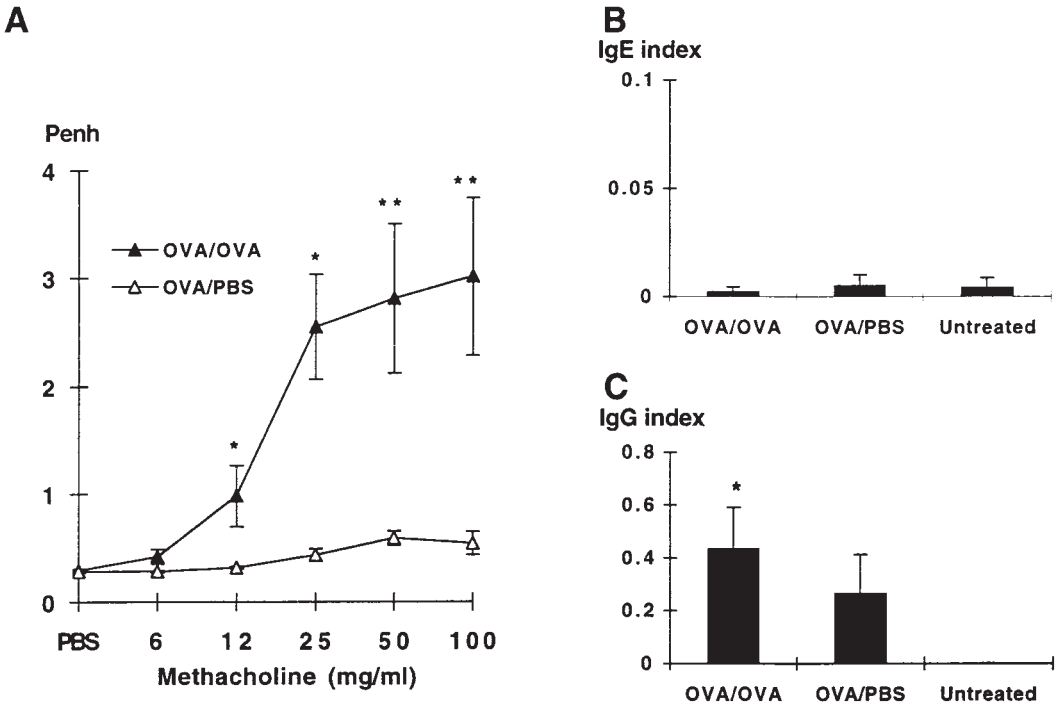

Figure 8. Repeat trial of 11 d exposure to aerosolized allergen (OVA) or PBS control. Mice rested for 2 wk after an initial airway sensitization protocol to OVA allergen (Figure 1) were subjected to a second trial to determine whether induction of tolerance occurred during the first trial. After the second exposure to OVA, significant AHR was seen in the OVA/OVA mice but not in the OVA/PBS controls (*A*). **$p < 0.01$, *$p < 0.05$ versus OVA/PBS; $n \geq 6$ mice/group. Serologic studies showed no significant OVA-specific IgE (*B*), but showed significantly increased OVA-specific IgG (*C*) after a second airway sensitization exposure (OVA/OVA). *$p < 0.05$ versus both other groups; $n \geq 6$ mice/group.

TABLE 4

BRONCHOALVEOLARS LAVAGE CELL FINDINGS AFTER SECOND AIRWAY SENSITIZATION PROTOCOL

	OVA/OVA	OVA/PBS
Total cells, $\times 10^5$/ml	0.61 ± 0.06*	0.39 ± 0.03
Macrophages, %	90.27 ± 1.75*	97.91 ± 0.03
Lymphocytes, %	3.68 ± 0.61†	1.08 ± 0.72
Neutrophils, %	1.86 ± 0.33	0.83 ± 0.36
Eosinophils, %	4.18 ± 1.17*	0.25 ± 0.25
Body weight, g	13.87 ± 1.14	14.07 ± 0.48
n	12	6

Definition of abbreviations: OVA = ovalbumin; PBS = phosphate-buffered saline.
* $p < 0.05$.
† $p < 0.01$.

airways of these very young mice at this time point. Similarly, no significant increase in Ia^+ cells was seen in the OVA group. The data show that coexposure to allergen and ROFA-s stimulated an influx of active immune cells, which might facilitate sensitization to allergen. The potential mechanisms for this recruitment remain to be investigated.

A number of limitations of this study merit discussion. First, the leachate from ROFA is not representative of all components present in particulate air pollution (31, 32). Nevertheless, soluble components of PM_{10} samples, as well as ROFA, can cause lung inflammation and injury when instilled into the lungs of experimental animals (8, 31). Use of ROFA-s offers practical advantages over the use of ambient air particles for initial experimentation. It is available in large quantities, allowing repeated experiments with the same material, and it can easily be aerosolized. Second, the dose of ROFA-s used experimentally (defined functionally as one that results in mild inflammation reflected in BALF at 24 h after exposure) is much greater than typical ambient exposures. Future experiments, using more realistic pollutant exposures (e.g., concentrated ambient air particles) and dose–response analysis for varying concentrations, are warranted by these initial findings.

Other experimental studies have also found that air pollution-related particles can act as immune adjuvants. Experimentally, it has been found that diesel exhaust particles can act as an adjuvant for enhanced response to allergens in animal models of asthma (11, 12). Fujimaki and colleagues (33), noting the high incidence of autoantibodies and rheumatoid arthritis in coal miners, observed that coal fly ash may also act as an adjuvant in a mouse intratracheal instillation model. Other investigators have used systemically sensitized animals to examine whether air pollution particles stimulate immunologic responses, especially T-helper 2 cell activation and immunoglobulin production (11, 34).

The results reported here support the theory that air pollution components may act as adjuvants in immune sensitization to airborne allergens during the neonatal-to-juvenile period. These experimental findings contrast with the failure of some epidemiologic studies to show a correlation between higher air pollution levels and higher prevalence of asthma (9). However, confounding factors (e.g., differences in rates of infection (2), and a surprising homogeneity in respirable particle ($PM_{2.5}$) levels across seemingly dissimilar geographic areas (35), preclude a definitive conclusion about a sensitizing role of air pollution components based solely on epidemiologic data. Since other injuries to the airways (e.g., from viral infection or cigarette smoke) are thought to be capable of promoting the initiation of asthma, it is reasonable to postulate that air pollutants may act in a similar manner under some conditions. The model described in this report will be useful for further studies to test this postulate.

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